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14. ABSTRACT
Interest in developing androgen receptor (AR) inhibitors with novel mechanism of action is slowly increasing since commercial anti-androgens (Bicalutamide, Flutamide, Nilutamide and Enzalutamide) face therapeutic limitations. Current therapies fail over a period of time because they all target hormone binding pocket on AR to which the receptor has already developed effective resistance mechanisms. One of the promising strategies to combat drug resistance is to develop the inhibitors that target an alternative binding pocket of the AR, called Binding Function 3 (BF3). In the current study, we report indole chemical series, identified through systematic *in silico* screen, as leading AR BF3 inhibitors. The most potent inhibitor (compound VPC-13566**)** demonstrated excellent anti-androgen potency, anti-PSA activity and abrogates androgen-induced proliferation of LNCaP and Enzalutamide-resistant prostate cancer cell lines. Moreover, it reduces the expression of AR dependant genes more effectively than current gold-standard Enzalutamide. These findings provide evidence that targeting AR BF3 pocket using small molecule inhibitors is a viable therapeutic approach for patients with advanced prostate cancer.

15. SUBJECT TERMS
Prostate cancer, small molecule drugs, androgen receptor, chemical genomics, drug resistance, hormone resistance,computer-aided drug design

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INTRODUCTION: Androgen receptor (AR), a member of the nuclear hormone receptor (NHRs) is a ligand-dependent transcription factor (1) with significant therapeutic relevance in prostate cancer (PCa) (2). Conventional AR-based therapeutics have mainly focused on targeting the traditional hormone binding pocket of the receptor (3;4). However, over a period of time, therapeutic efficacy of these drugs suffered from the problem of drug resistance (5) due to 1) mutations at the ligand binding pocket causing structural and functional changes of the receptor and 2) conversion of antagonist into an agonist in a physiological context. Antiandrogen-based therapy including second generation androgen receptor (AR) inhibitors, Enzalutamide represents a typical case, where drug resistance is inevitable and its onset is associated with poor prognosis and high mortality (6). This highlights the urgency to develop entirely new types of anti-AR therapeutics with novel mode of action *i.e.* rather than blocking ligand interaction with AR the novel class of drugs should ideally bind to alternative sites on the receptor thereby effectively disrupting the association of coactivators that bind to these sites. Recently, a co- regulatory surface site called the Binding Function 3 (BF3) has been identified by Fletterick *et al* (7). The specific function of this pocket has not yet been fully characterized, although recent reports provide some evidence of its possible involvement in the AR association with FKBP52 - an important positive regulator of this receptor (8) and possible cross-talk with the AF2 site (9). In addition, BF3 is conserved among other members of NHR family, thereby offering a better opportunity to understand the molecular mechanism of co-factor recruitment and subsequently its inhibition. Since AF2 and BF3 surface pockets play pivotal role in mediating AR function, therapeutic targeting of these sites offers a rich vein for the discovery of novel drugs for PCa with alternative mechanism of action and thereby circumvents treatment resistance seen with conventional anti-androgens. These drugs would provide an additional line of therapy for patients combating castration-resistant PCa thereby considerably expanding their life span.

BODY:

Specific Aim 1: to use the generated structure-activity data and the resolved crystal structure to design and synthesize AR AF2 and AR BF3 binders with enhanced target-affinity and 'drug-like' properties.

Task 1.1. Molecular modeling of derivatives of our lead AF2binders VPC-0061 and lead BF3 binders VPC-0098 and a closely related VPC-4035.

In order to enhance the activity profile of our BF3 leads, structure based lead optimization was carried out on 2-((2-phenoxyethyl) thio)-1H-benzo[d]imidazole in a previous study (10). The resulting derivatives demonstrated decent anti-AR activity in low micro molar range and inhibit mutated/agonist form of AR in LNCaP cell lines including those which have developed resistance to the recently approved drug, Enzalutamide. In addition, these derivatives significantly reduced PSA levels compared to Casodex in Enzalutamide resistance cell lines. The only limitation of the study on benzoimidazole series was we did not achieve great potency that could potentially reflect *in vivo* applications. One possible explanation was that synthetic derivatives with long linkers, connecting two aromatic ring systems did not achieve decent potency. In the present work, on the basis of reported benzoimidazole derivatives, we searched for chemicals containing different types of short linkers using 2-[(2-phenylethyl) sulfanyl]-1H-1,3-benzodiazole as template to yield various analogues with enhanced potency.

ROCS (Rapid Overlay of Chemical Structures) (11), a shape-based similarity method based on molecular volume, from OpeyEye was employed to search through ZINC database v12.0 (12). All software parameters were set to their default values. The search resulted in 10 analogues. The resulting compounds were mapped to the query template and ranked according to the "Shape Tanimoto (Shape Tc)". The structures were inspected visually and only one compound that interestingly met our criteria (*i.e.* short linker connecting two aromatic systems; Compound 2) and having Shape Tc above 0.8 with respect to the query structure was selected and tested for its anti-AR activity. Since the compound 2 represents novel chemical prototype (3-[(E)-2-phenylethenyl]-1H-indole), it was used as a chemical input to identify next chemical series (N-[1H-indol-3-ylmethylidene] aniline; compounds 3-11).

Similarly, each subsequent chemical series (3-[(E)-2-phenyldiazen-1-yl]-1H-indole; 3-(3H-indol-2-yl)-1H-indole; 3-(2, 3-dihydro-1H-indol-2-yl)-1H-indole) was identified based on the scaffold of active compounds from the previous series (15). From each series we obtained different number of compounds, which sum up to 500 analogues. These compounds were further evaluated using our established *in silico* pipeline.

Molecular Docking of Selected Compounds into AR BF3 Pocket

The BF3 site represents a hydrophobic groove located adjacent to AF2 pocket on the surface of AR. Being a protein-protein interaction site, the BF3 is a challenging target, nevertheless, it offers an attractive option of direct inhibition of the AR transactivation. Using our *in-house* computational drug discovery pipeline we virtually tested 500 compounds, selected previously. Our *in silico* pipeline included molecular docking, on-site rescoring, and consensus voting procedures (as explained in the Materials and Methods).

To begin with, all the structures were docked into the AR crystal structure (4HLW with 2.5 Å resolution) using Glide SP program (13). Our previous study confirmed that the charged amino acid Glu837 and Leu830 forms a H-bond interaction and hydrophobic contact with BF3 inhibitors, respectively and are critical for binding. Therefore, we applied H-

bond and hydrophobic constraints in the BF3 site during docking. Compounds that received moderate to higher score by Glide SP were selected and re-docked into the 4HLW structure using the eHiTS docking protocol (14). In order to improve the accuracy of the predicted binding orientation of the compounds, the root-mean-square deviation (RMSD) was calculated between the docking poses generated by Glide and eHiTS. Only molecules with docking poses with RMSD values below 2.0 Å were subjected to further analysis.

In the next step, selected docked ligands were subjected to additional on-site scoring using the Ligand Explorer (LigX) program and the p*Ki* predicting module of the Molecular Operating Environment (MOE) (15). With this information, a cumulative scoring of four different predicted parameters (Glide score, eHiTS score, LigX score and pKi predicted by the MOE) were generated with each molecule, receiving a binary 1, 0 score for every "top 20% appearance". The final cumulative vote resulted in about 62 compounds that consistently demonstrated high predicted binding affinity toward the BF3 site. These compounds were then visually inspected and on the basis of their commercial availability we purchased 23 compounds (compound 2-24; Figure 1) to evaluate in our cell based screening.

Task 1.2. Synthesis of derivatives of our lead AF2binders VPC-0061 and lead BF3 binders VPC-0098 and a closely related VPC-4035.

Based on these findings, 422 small molecule compounds were synthesized either at Enamine **(http://www.enamine.net/)** or at our collaborator Robert Young's laboratory and send to the VPC for further testing (see Specific Aim 2).

Specific Aim 2: to experimentally evaluate the developed synthetic derivatives.

Task 2.1. eGFP Cellular AR Transcription Assay.

All the selected compounds were screened for their ability to inhibit AR transcriptional activity using a nondestructive, cell-based enhanced green fluorescent protein (eGFP) AR transcriptional assay (16). In this assay, the expression of eGFP is under the direct control of an androgen responsive probasin-derived promoter and enables quantification of AR transcriptional activity. All compounds that exhibited >75% inhibition of AR transcription at a screening concentration of 3μM were then subjected to concentration-dependent titration to establish their corresponding IC_{50} values (Figure 1). To ensure these values are true positive hits in the AR transcriptional eGFP assay, we validated their activity by a second transcription assay, based on light detection instead of fluorescence, by quantifying their effect on the production of the prostate specific antigen (PSA) in prostate cancer cell lines (17). PSA is AR-regulated serine protease and is widely used as a biomarker for PCa. As expected, hit compounds induced a equivalent decrease in PSA levels in LNCaP (18) cells as the IC_{50} values found with the eGFP assay. Table 1 below summarize compounds that were found to have an IC50 below 1 μM. For comparison purposes, in this assay, gold standards Enzalutamide and Bicalutamide shows IC_{50} of 100 nM and 600 nM respectively. Importantly, two compounds exhibit exceptional activity under 60 nM. Figure 1 shows a typical experiment for determination of IC_{50} (in this case compound VPC-13163) using Enzalutamide (MDV3100) as comtrol.

Table 1: Compounds that exhibited an IC_{50} under 1 μM in transcription assays (eGFP and PSA)

VPC-#	eGFP IC50 (μM)	PSA IC50 (μM)
13566	0.004	0.011
13562	0.06	0.14
13226	0.11	0.12
13163	0.31	0.21
13259	0.33	0.25
13521	0.37	0.26
13256	0.4	0.5
13256	0.42	0.13
13541	0.51	0.94
13255	0.52	0.4
13554	0.59	0.67
13127	0.6	0.5
13537	0.66	0.8
13276	0.71	0.5
13534	0.81	0.99
13247	0.91	0.45

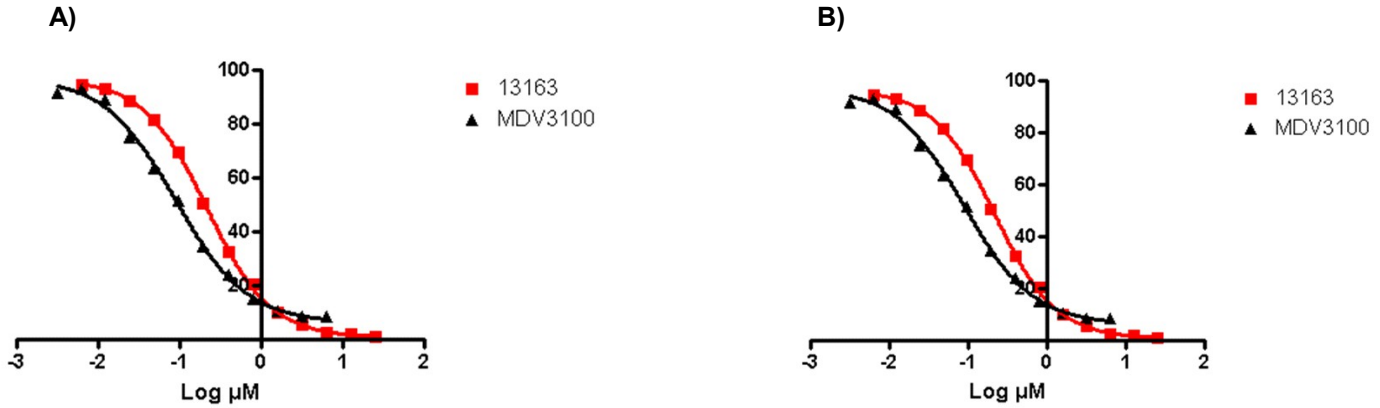

Figure 1: Sigmoidal curves of compound VPC 13163 to determine IC50 in A) eGFP and B) PSA. The Y-axis represents the percentage of activity remaining after treatment with the compound.

Task 2.2. *MTS assay.*

To determine the translational potential of the most potent BF3 inhibitors listed in Table 1, we evaluated their ability to reduce growth of PCa models stimulated by the androgen R1881 *i.e.* LNCaP, and androgen-independent PC3 cell line. The cell viability was assessed after 4 days of incubation with the test compound in a concentration dependent manner. Figure 2 shows a typical experiment with our lead compound VPC-13566 where the compound is very effective in inhibiting the growth of LNCaP cells, establishing an IC_{50} values of 100 nM. Moreover, compound 131566 did not show any effect on AR independent PC3 cell lines, confirming its AR-specific activity. MTS experiments are performed on all our compounds that are under 1 µM in eGFP activity.

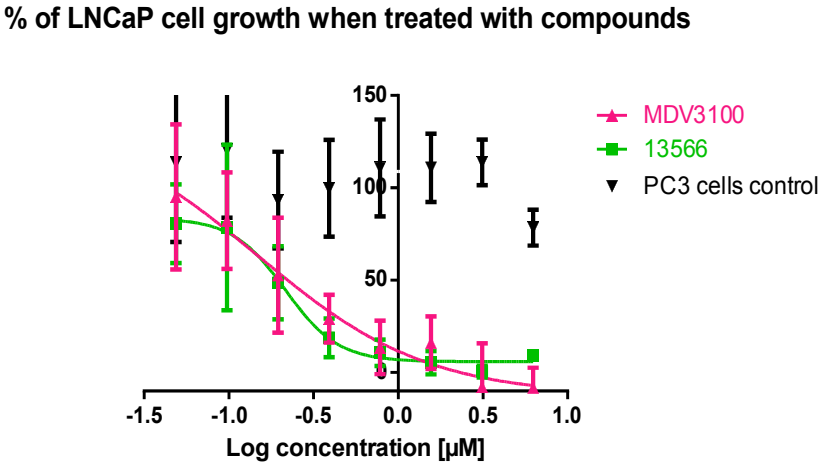

Figure 2: The effect of compounds VPC-13566 on cell viability in LNCaP and PC3 cells. In all our MTS experiments Enzalutamide (MDV3100) is used as control.

Since this compound is very promising for a potential alternative therapy when the current gold standard anti-androgen therapy fails, we tested the ability of this compound to reduce the growth of a cell line developed in-house that

is resistant to Enzalutamide. As can be observed in Figure 3, our lead compound VPC-13566 can clearly inhibit the growth of Enzalutamide-resistant cells, at an IC50 similar to what was observed in LNCaP cells.

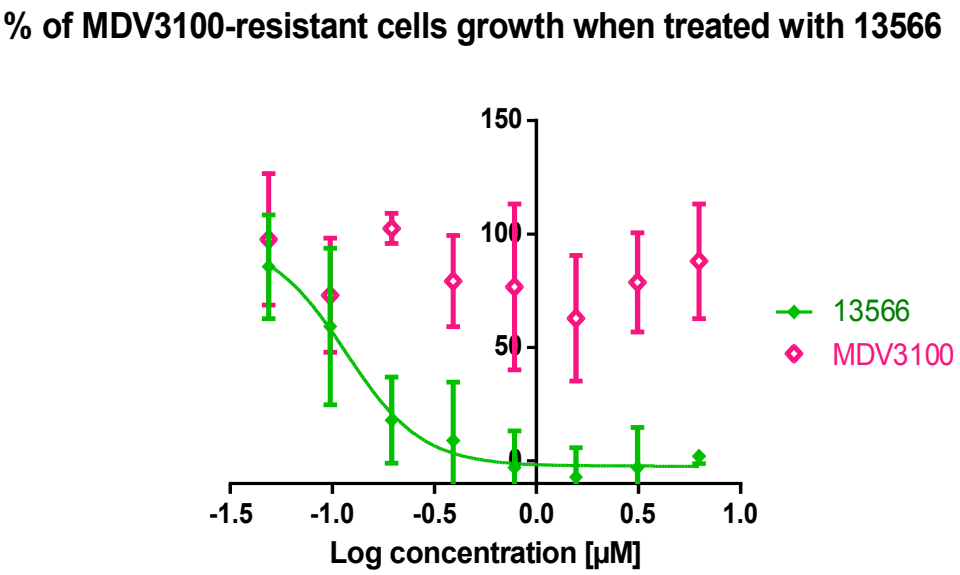

Figure 3: The effect of compounds VPC-13566 on cell viability in MR49F cells, an Enzalutamide-resistant cell line. Enzalutamide (MDV3100) is used as control.

Task 2.3.*Biolayer interferometry*.

Biolayer interferometry (BLI) studies demonstrated a direct reversible interaction between these compounds and a purified AR ligand binding domain in a dose dependent manner. When a compound binds to the AR, there is a shift in wavelength that is detectable and measurable using the instrument. Figure 4 shows that the BLI data for the highest active compound **13566**. There is a clear binding pattern for this compound to the AR. All BF3 compounds exhibited this behavior with more or less efficiency depending of the potency of the compound. As a further task in 2.3 we also tested the ability of the compound to displace a peptide that bind to an adjacent site (AF2) but none worked, suggesting that binding of our BF3 compounds does not occur to alternate sites such as the AF2 (not shown)

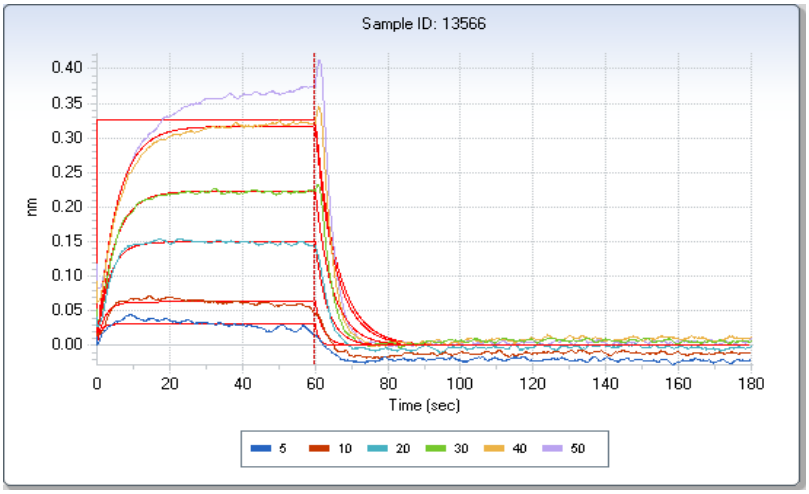

Figure 4: Binding of the VPC-13566 compound to AR determined by Biolayer Interferometry analysis

Task 2.4. ***Protein expression, purification, crystallization and data collection.***

This part of the work is still in progress. On the second year of this project we will submit our most potent inhibitor VPC-13566 for crystal structure analysis.

Specific Aim 3: to select several lead compounds for pharmacological development.
Task 3.1. *Solubility, Stability and formulation for in vivo studies.*

This part of the work is scheduled to start this year. We will evaluate our strongest compounds for their pharmacokinetic evaluation, stability, toxicity, efficacity in our mice model. Preliminary data using microsomal stability has shown that our compounds are not great stability-wise, but we plan to investigate this further with animal work as well as to try to improve the stability of the lead compounds using further medchem optimisation.

KEY RESEARCH ACCOMPLISHMENTS: Bulleted list of key research accomplishments emanating from this research.

- Uding our in-silco model we identified and characterized 16 small molecule compounds inhibitor of the AR with an IC50 under 1 μM in transcription assay.
- Two of these compounds exhibit exceptional activity; VPC-13562 is equivalent to gold standard Enzalutamide while VPC-13566 is 5 to 8 times more efficient than Enzalutamide.
- These compounds show a direct interaction with the AR by BLI and are effective to reduce the growth of Enzalutamide-resistant cell lines
- Based on these outcomes, it can be concluded that BF3 specific inhibitors can act as complementary therapeutics to treat castrate-resistant prostate cancer

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

Infomatics such as databases and animal models, etc.;
We created a chemogenomic database of genes differentially expressed when LNCaP cells are treated with our compound VPC-13163. This may lead to successful investigation of the complete mechanism of action of these powerful inhibitors.

CONCLUSION:

Prostate cancer is the most commonly diagnosed non-skin cancer in Canadian men and one of the leading causes of cancer-related death. If diagnosed early, when still confined to the prostate, it is frequently curable by surgery or radiotherapy. Treatment for locally advanced, recurrent or metastatic prostate cancer is primarily some form of androgen withdrawal therapy, which is generally designed to block either the production of androgens or their binding to the androgen receptor. Unfortunately, the effectiveness of this type of treatment is usually temporary due to progression of surviving tumor cells to a castration-resistant state. With no curative treatment options for castration-resistant prostate cancer, the median life expectancy is approximately 18 months. Part of the problem is that all anti-AR agents currently used to treat patients act by direct binding to the AR hormone binding site and hence are vulnerable to mutations which frequently arise in this region of the molecule. The proposed research aims to address this problem by using computer modeling, biological screening, and structural biology to develop an entirely new class of anti-AR drugs which will target an distinct region of the AR called binding function-3 (BF3) to inhibit its activity. We anticipate that these new anti-AR drugs will replace or supplement existing anti-AR therapeutics and will provide new options for treating patients with metastatic, castration-resistant prostate cancer.

Over the last year, there has been considerable progress in our project to develop small molecules inhibitors that target the BF3 site of the AR for the treatment of prostate cancer. Based on the chemical scaffold (benzoimidazole) of previously reported BF3 inhibitors (10), we conducted a systematic *in silico* screen and identified approximately 200 indole based compounds for biological testing. These compounds were evaluated successfully using a series of *in vitro* assays confirming their potency via AR guided mechanism of action in various prostate cancer cell lines. Several compounds demonstrated inhibition of AR in low micro-molar range. One of the most potent inhibitors initially identified in that first wave of modelling, VPC-13163, demonstrated an IC_{50} of 0.31μM in AR eGFP transcriptional assay. Confirming it as a true BF3 binder, VPC-13163 neither displaced the co-activator from an alternative coactivator binding site, AF2, nor androgen from the Androgen Binding Site. Based on the success of these computational and biological approaches, a second wave of derivatives of VPC-13163 were synthesized by our collaborators and thus, hundreds of additional AR inhibitors were evaluated for their AR inhibition capabilities. A chemical transition of VPC-13163 resulted in two synthetic derivatives which demonstrated inhibition activity in low nano-molar range. VPC-13562 and 13566 exhibit an IC_{50} of 0.061μM and 0.004μM in AR eGFP transcriptional assay and IC_{50} of 0.14μM and 0.011μM for PSA inhibition, respectively.

It should be noted that these derivatives show 5-80 fold increase in the activity when compared to their compound VPC-13163. Additionally, the Biolayer Interferometry (BLI) assay detected direct reversible interactions between the AR and VPC-13566. This class of inhibitors is promising and is currently under further investigation. Importantly, VPC-13566 exhibited a strong anti-proliferative effect on both LNCaP and Enzalutamide-resistant cell lines (MR49F) with no effect on the AR independent PC3 cells, confirming that mutations in the hormone binding pocket do not affect the efficacy of VPC-13566. VPC-13566 inhibits prostate specific antigen (PSA) in both LNCaP and MR49F and reduces expression of AR target genes (PSA and TMPRSS2). Based on our outcomes using original scaffold and an alternative binding pocket BF3, it can be concluded that BF3 specific inhibitors can act as complementary therapeutics to treat castrate-resistant prostate cancer.

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